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(71) Applicant *(for all designated States except US)*: ISIS INNOVATION LIMITED [GB/GB]; 2 South Parks Road, Oxford OX1 3UB (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : FRANCIS, Martin, James, Ogilvie [GB/GB]; 13 Sandfield Road, Headington, Oxford OX3 7RQ (GB). BERESFORD, Jon, Nicholas [GB/GB]; 64 West Street, Chipping Norton, Oxfordshire OX7 5ER (GB). GRAVES, Stephen, Ellis [AU/AU]; 38 Rose Terrace, Wayville, Adelaide, S.A. 5034 (AU).

(74) Agent: PENNANT, Pyers; Stevens, Hewlett & Perkins, 1 Serjeants' Inn, Fleet Street, London EC4Y 1LL (GB).

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(57) Abstract

A method of maintaining bone and bone-forming cells in culture involves providing the cells, at the primary culture stage, with a long-acting source of ascorbate such as L-ascorbic acid 2-phosphate. Preferably the cultured cells, at the first passage stage or subsequently, are introduced into the body as an allograft or an autograft or a xenograft, with the support of a biocompatible matrix or in the form of a suspension.

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CULTURE OF BONE CELLS

The present invention is concerned with the culturing of bone cells.

BACKGROUND OF THE INVENTION

Bone is a specialised connective tissue. It provides support and protection for other tissues of the body, allows movement and functions as an organ in mineral and haemopoietic homeostasis. It contains a large number of cell types and a unique intercellular matrix. Some of the cells are specific to bone and are responsible for its development, maintenance and repair. The matrix contains both organic and inorganic components. The organic component consists chiefly of collagen, but also contains a large number of other organic molecules. The inorganic (or mineral) component is a poorly crystalline carbonate-containing analogue of hydroxyapatite. There is an intimate relationship between cells, organic matrix production and the deposition of mineral that results in the formation of bone.

Ascorbic acid (Vitamin C) is one factor known to be important in the normal function of human bone forming cells (osteoblasts). Unlike most other mammals humans do not synthesise ascorbic acid but are entirely dependent on dietary ascorbate to supply their requirements. In scurvy, which is due to a deficiency of ascorbate, bone formation completely ceases.

Despite the apparent importance of ascorbic acid to *in vivo* bone formation there are few reports on its effects on bone-derived cells *in vitro*. In foetal rat bone organ culture, ascorbic acid was shown to increase collagen, but not non-collagenous protein

synthesis. Mineralisation was also not affected, but was deposited as a calcified cartilage core (Chen and Raisz, 1975). Ascorbic acid is known to be essential for mineralised matrix production by bone-derived cells in vitro (Anderson et al, 1984; Aronow et al, 1990; Bellows et al, 1986; Nefussi et al, 1985; Ecarot-Charrier et al, 1983; Tenenbaum and Heersche, 1982).

It is reported to promote osteoblastic differentiation (Spindler et al, 1989; Sugimoto et al, 1986). In a study using a relatively undifferentiated human osteosarcoma cell line (MG-63) ascorbic acid inhibited cell growth but increased alkaline phosphatase activity and enhanced the stimulation by $1,25(OH)_2VitD_3$ on alkaline phosphatase activity (Franceschi and Young, 1990). In contrast, stimulation of proliferation of the osteoblastic cell line MC3T3-E1 and normal rat bone-derived cells has been reported (Harada et al, 1990; Spindler et al, 1989). It has also been shown to stimulate the extracellular biosynthesis of matrix vesicles (Wuthier, 1988).

The role of ascorbic acid in collagen synthesis is well established (Peterkofsky, 1972) although its mechanism of action is not fully understood. The importance of ascorbic acid to bone formation has largely been attributed to its effects on collagen synthesis. The histological studies of bone in scurvy indicate that osteoblast proliferation, differentiation and viability are affected. The extent to which these effects are consequent upon collagen synthesis or some other alternative effect or effects is uncertain.

Disorders of bone formation present major problems in Orthopaedic practice. Ost oporosis, fracture nonunion, prosthetic loosening and the replacement of large defects in bone as well as

heterotopic bone formation are common but difficult clinical problems. To improve the management of these conditions the physiological mechanisms involved in bone cell differentiation and matrix production which remain largely unknown need to be understood. However bone is a complex and difficult tissue to study. In an attempt to provide a more isolated and controlled environment, bone cell tissue culture systems have been developed. The majority of these systems have used bone cells obtained from animals however their relevance to human bone cell physiology is not known. Studies involving human cells have been much less frequent. This is in part due to the difficulty of obtaining sufficient growth of suitably differentiated cells using conventional techniques when compared to the animal models. There are a large number of cell lines now available expressing different features of the osteoblastic phenotype. These have the advantages of availability and convenience but they do not represent the normal range of phenotypic expression in bone. Although they may express tissue specific functions that may be relevant to the normal osteoblast the variety of responses that they are capable of is reduced by their limited phenotype. This invention focuses on the development of improved techniques for culturing normal human bone cells.

There is a very large and increasing number of patients that would potentially benefit directly from such developments. These include patients with fracture nonunion, defects in bone secondary to trauma or surgical resection, joint fusion and the management of failed total joint replacement. This latter area has become one that is increasingly important. All current joint replacements eventually fail and the vast majority fail within 10-15 years. This failure results

from bone loss around the prosthesis which allows it to loosen. Unless this bone is restored a revision replacement fails within a very short time. The large number of joint replacements already done means that there is a rapidly increasing number of people that require extensive bone reconstruction.

The current methods of treatment for all of these problems including reconstruction after failed joint replacement have been to use either bone taken from another part of the patients body (autologous graft) or donor bone from another human (allograft) or mammal (xenograft). There are serious problems associated with both of these methods.

The use of autologous bone is the best alternative but it has three main problems. The first is that there is a limited supply so it can only be used in treating small areas of bone loss. The second is its failure to provide structural stability. This is because only small fragments of mainly trabecular bone are obtained when harvesting and in many situations it is not possible to achieve the structural stability using these fragments. The third is that there is considerable morbidity to the patient when bone is taken from other areas of the body.

Allografts on the other hand have been particularly useful because they provide the structural stability not possible to achieve with autologous grafting. Whole bones or large segments of bone can be completely replaced using allografts. Unfortunately the bone is dead and there is no inherent bone forming property within it. It does however, act as a lattice on which the patient can form their own bone. While this is happening the allograft is also slowly being reabsorbed. This replacement process however takes many years and is never completely achieved and as a

consequence the strength of the bone never reaches a normal level. There are many other problems associated with allografting. Its use is limited to centres which have bone banking facilities. There is a 50% major complication rate, largely due to infection and fracture. A.I.D.S. transmission has become a serious risk. In an attempt to lessen this risk many centres are now irradiating the bone which considerably weakens it, thereby decreasing its structural stability advantage. In addition several patients have recently developed leukaemia after grafting. The future of allografting is now very bleak and there is a growing opinion that it should be abandoned altogether.

The limitations of these methods mean there is no satisfactory solution to the problem of a rapidly increasing number of patients requiring bone reconstruction.

One solution is the development of artificial materials which can act as a bone substitute. For a biomaterial to be an effective bone substitute it is necessary that it bonds directly to the remaining bone and allows new bone to grow on its surface. This requires that the material is not only compatible with bone but that it also provides a surface that promotes bone cell attachment, differentiation and matrix formation in preference to other cell types. The methods used to determine these effects largely involve the in vivo assessment of these materials by a variety of animal models. This is not only expensive and time consuming but may not be (and often is not) relevant to the human response. A solution to this problem would be a reliable and rapid in vitro method of assessing the response of human bone forming cells to these materials.

The in vitro expansion and reimplantation

of a patient's own bone is another way of dealing with bone loss. This kind of technique has already been used for a number of other cell types. In the treatment of patients with severe burns and insufficient normal skin to use as grafts small amounts of the remaining normal skin have been removed, expanded in vitro on a variety of matrices and then used to graft the burn areas. The recent reporting in the public press of implanting donor pancreatic cells for the treatment of diabetes mellitus is another example.

Again, however, as well as the problems with culturing human bone and bone-forming cells, there has up till now been no reliable method for in vitro assessment of materials to develop a suitable matrix for the purpose of bone reimplantation.

The conventional techniques for the culture of human bone cells do not use ascorbic acid unless studies requiring matrix production are being undertaken. Na ascorbate is the usual form of ascorbate used in these situations. In cell culture conditions it is unstable, having a half life of 7-10 hours. As culture medium is changed every 2-3 days the addition of Na ascorbate results in a pulsed treatment of the cells. Under normal in vivo circumstances there is a constant level of ascorbate, therefore the use of Na ascorbate cannot be regarded as physiological. The effects of a long acting form of ascorbic acid (L-Ascorbic Acid 2-Phosphate) on the culture of human skin fibroblasts has been published (Hata and Senoo, 1989). It was reported that in culture the compound remained stable for one week and had beneficial effects on both cell proliferation and matrix production.

The structure of ASC-2P is shown in Figure 1.

The earliest stage in culturing bone and

bone-forming cells, that is, when the cells and/or bone explants are first placed in culture, is known as primary culture. First passage cells from bone are the cells derived from bone explants or other bone cell primary cultures typically after four to six weeks in primary culture.

First passage cells from bone have been shown to benefit from presence of a long-acting form of ascorbate (Graves et al, 1990). Markers of osteoblastic differentiation and the matrix produced were enhanced by the addition of ASC 2P compared to sodium ascorbate.

DESCRIPTION OF THE INVENTION

Previous techniques have not involved providing primary cultures of bone and bone-forming cells with a source of ascorbic acid. It has been found that the continual presence of ascorbate in the culturing of bone cells is an important improvement in technique. The present invention therefore provides a method of maintaining bone and bone-forming cells in culture wherein the cells are provided with a long acting source of ascorbate from the primary culture stage.

Bone and bone-forming cells include marrow stromal osteoprogenitors and may come from the bone marrow, bone and periosteum (the fibrous layer covering the outer surface of the bone).

Although the invention is of primary interest for human bone and bone-forming cells, it also extends to the cells of other animals.

In the initial culture of bone cells, it is necessary to change periodically the culture medium, e.g. to keep reagent concentrations steady. But every change disturbs the cells, is labour- and time-

consuming and gives rise to risk of infection. Changes of culture medium more than about once a day would not be practicable. Changes every 2-3 days are preferred.

Sodium ascorbate could not be used in these cultures as a source of ascorbate because of its short half-life and because of the risk of building up toxic concentrations of decomposition products. A long-acting source of ascorbate is one which provides a continuing supply of ascorbate in a form usable by the cells, at a practicable frequency of culture medium change. The preferred long-acting source of ascorbate is L-ascorbic acid 2-phosphate (ASC 2P), a commercially available compound.

The nature of the culture medium is not material to the invention, and conventional media can be used. The ascorbate concentration in the fresh culture medium as added should preferably be in the range 2-2,000 μM, and the ascorbate concentration in contact with the cells should preferably not fall below 2 μM preferably 20 μM.

Histological studies show that explants cultured in ASC-2P retain more normal appearance of the stromal elements of bone including well-differentiated cells producing matrix. Explants cultured with ASC 2P show poor matrix formation with fewer numbers of cells and poor osteoblastic differentiation (i.e. the absence of ascorbate has a detrimental effect on the proliferation and differentiation of the stromal cells within bone).

Crossover studies also give important results. These involve culturing explants with or without ASC 2P. Cultures of passaged cells from each group are then continued, either with or without ASC 2P, so that four groups of differently treated cells are obtained. On examination of these, cells

from explants without ASC 2P show increased cell proliferation and matrix production as well as osteoblastic differentiation when they have been continued in the presence of ASC 2P, compared to those continued with no ASC 2P.

When cells from explants cultured in ASC 2P are continued in ASC 2P they show a further proliferative capacity and matrix production. However, this group shows a loss of osteoblastic differentiation which is then found to be inducible by factors known to promote it in vitro. Subsequent studies show that following induction and the addition of factors known to promote mineralisation in vitro for animal bone derived cells, the cells from the ASC 2P treated explant group continued in ASC 2P mineralise. There is little or no mineralisation in the cells from explants not treated with ASC 2P even if ASC 2P is added at the time of the first passage.

In addition, in conditions known to promote adipocytic differentiation in bone cells derived from animals, adipocytic differentiation develops only in the cells from explants cultured in ASC 2P. Adipocytic differentiation of cells derived from human bone derived cells has not been previously reported.

In the presence of ascorbate, not only is the normal arrangement of the stromal elements in the explants maintained, but also the populations of cells derived from explants are more proliferative, produce more matrix and have a greater capacity to react to factors influencing cellular differentiation and function. Although cell culture conditions inducing cell proliferation can be achieved using other substances influencing cells such as growth factors, the resulting cells are of a poorer quality and the cost of such methods can be substantial.

At the end of the primary culture period, when the cells have been separated from the bone fragments or other living material from which they were derived, the resulting first passage cells may be used in various ways. They may be further cultured in vitro, preferably in the presence of a long-acting source of ascorbate such as ASC 2P, under conditions which are not material to the invention and which may be conventional. The cells, either at the first passage stage or after further culturing may be implanted into a patient, or used in other ways.

The invention further provides a method of culturing bone and bone-forming cells wherein the cells are cultured in vitro with a long-acting source of ascorbate from the primary culture stage, and are subsequently implanted into the recipient as an allograft or an autograft or a xenograft.

The cells can be obtained by taking either a small amount of bone or alternatively bone marrow which is also a source of osteogenic cells. In the presence of a long-acting source of ascorbate the cells can be rapidly expanded over a 4-6 week period and then reimplanted. To ensure the bone-derived cells are retained in the appropriate area it may be necessary to implant them in a biocompatible (i.e. supports bone formation) matrix. This has the advantage of being able to shape the graft to the exact dimensions needed. If structural stability is required then the cells can be implanted into a structurally stable biocompatible material such as ceramic. A number of materials already developed would be suitable such as A-W glass ceramic. The rate of bone formation after implantation is likely to be rapid i.e. measured in weeks.

The advantage of the invention in this embodiment is that using a long-acting source of

ascorbate allows culturing of osteogenic cells to be much faster and they appear to be of much better "quality" than cells cultured using conventional techniques. There is another potential advantage in that in the presence of a long-acting source of ascorbate the cells produce a large amount of matrix. Bone matrix has the ability to induce non-osteogenic cells to differentiate into bone cells. So there is the two fold advantage of being able to implant large numbers of the patients own healthy bone cells with a significant amount of matrix which may promote recruitment of further bone cells from surrounding non-osteogenic tissue.

In some instances it may be useful to use cells grown up in this way as an allograft or a xenograft. Bone substitutes derived from animal bone are currently commercially available.

In another embodiment, the invention provides a method in which one or more materials are added to the cells cultured as above and the effect of the material or materials on the growth and condition of the cells is assessed.

To assess the effects of a biomaterial on bone cells cultured in ASC 2P from primary culture these cells can be co-cultured with the material in the presence of ASC 2P. Effects of the biomaterial on cell contact, proliferation, differentiation and matrix production can then be determined. Cellular contact can be assessed by light and electron microscopic examination. With the addition of well described specialised staining techniques the nature of the matrix formed can also be examined using these methods. In this manner it is possible to determine if any matrix formed is bone-like. In addition the standard techniques that have been used to assess cell

proliferation, and quantify the effects on cell differentiation and matrix production used in the original studies on the effects of ASC 2P alone can also be employed.

A-W glass ceramic, a material that has already been used for replacing bone in humans, is known to show useful properties as a biomaterial in vivo. Its effects in vitro on bone cells have now been investigated. Using conventional culture techniques the material is detrimental to human bone-derived cells. However using cells that have been cultured in ASC 2P from the explant stage there is promotion of osteoblastic differentiation and matrix production by the A-W glass ceramic.

Another material that has now been tested is the bioactive material that is the subject of our U.K. patent application GB 91 22 329.7. This is also shown to promote osteoblastic differentiation and matrix production when bone cells are provided with ASC 2P.

The methods used to assess these materials are distinguished only by the fact that the cells involved are cultured in ASC 2P from the explant stage. The cells therefore have the ability to react to osteoblastic stimuli in a much better manner than cells obtained using conventional techniques and are rendered more appropriate for use is assessing effectiveness of biomaterials.

EXPERIMENTAL

In order to establish the effects of long-acting ascorbate on bone-derived cells when added in primary culture, a crossover study was performed. Bone explants were cultured with or without a long-acting form of ascorbate and the effects were determined using first passage cells.

METHODS

Cell Culture

Culture Medium:

A standard culture medium was used for all cell culture and subsequent experiments. This was Dulbeccos modification of Eagles minimal essential medium (Dmem) prepared in ultra-pure water and further supplemented with:

1. $NaHCO_3$ 0.85 g/l,
2. HEPES 20 mM,
3. L-glutamine 0.292 g/l,
4. Benzyl penicillin 25 units/ml,
5. Streptomycin sulphate 25 µg/ml,

The pH was adjusted to 7.35 at 37°C by the addition of 10M NaOH. 10% v/v fetal calf serum was added before use.

Human bone-derived cells:

Human bone derived cells were obtained by outgrowth from explants of normal human trabecular bone using a modification of the method described by Beresford et al. (1983)

The bone was cut into small fragments 3-5 mm in diameter, washed by vigorous vortexing in calcium and magnesium free phosphate buffered saline (PBS) to remove blood and marrow. This wash was repeated three times. Eight to ten explants were then placed into T80 flasks containing 10 mls of culture medium and incubated at 37°C in an atmosphere of 5% CO_2 and 95% humidity. The medium was changed completely at 7 and 14 days. Thereafter the medium was completely changed three times a week (Mon, Wed and Fri). The culture medium in half of the flasks was supplemented with L-Ascorbic Acid 2-Phosphate at 100 µM concentration throughout. The cells were maintained for four weeks

after which time they were passaged.

Cell Passage:

At the time when they were passaged, the cells cultured in standard medium had almost reached confluence. The cells in flasks supplemented with ASC 2P had reached confluence 7 days earlier and at four weeks were multilayered.

After removal of the explants some of which were prepared for histology, cells from both groups were passaged using a standard trypsin/EDTA method, and counted. The presence of a large amount of matrix in the ASC 2P treated primary cultures resulted in clumping of the cells when passaged using a trypsin/EDTA solution alone. This problem was overcome by pretreating the cultures with 250 units/10 ml SFM of clostridial collagenase for two hrs prior to trypsinisation. The pretreatment with collagenase did not result in any cell separation during that time. This method of disaggregation was used for all culture conditions in these studies.

Cells were counted and resuspended at the required density, calculated using viable cells only (cell viability was never less than 90%).

Crossover Studies: The cells were plated at a density of 2.5×10^4 cells/well into 24 well multiwell culture plates and a crossover study was performed by continuing cells from both primary culture conditions in either standard medium or standard medium supplemented with ASC 2P. In this manner 4 groups were established;

1. Cells cultured in standard medium in primary culture and continued in standard medium in secondary culture (SS).

2. Cells cultured in standard medium in primary culture and changed to standard medium supplemented with ASC 2P in secondary culture (SA).

3. Cells cultured in standard medium supplemented with ASC 2P in primary culture and then changed to standard medium in secondary culture (AS).

4. Cells cultured in standard medium supplemented with ASC 2P in primary culture and continued in standard medium supplemented with ASC 2P in secondary culture (AA).

The cells were then cultured for a further seven days and the media changed every two days.

The parameters tested were: cell proliferation assessed by cell counts, total DNA and by thymidine incorporation, total protein content, alkaline phosphatase activity, osteocalcin release, collagen and non-collagenous protein synthesis.

For investigations involving the addition of radioactive labelled precursors, these precursors were added at the time of the media change on the sixth day.

In a separate series of studies the effects on alkaline phosphatase activity of a 24 hr exposure to 10^{-8} M 1, 25-OH_2 Vitamin D_3 plus 10^{-8} M Vitamin K (D+K) and 10^{-8} M dexamethasone (DEX) either alone or in combination with D+K was also examined. The cells were cultured as described above and these additives were included in the medium on the 6th day.

Assessment of Cell Cultures

Cell Growth:

Three methods were used to assess cell growth. Apart from cell counting which was confined to assessing cell growth in culture flasks two other methods were used for cells cultured in 24 well multiwell culture plates. These were the determination

of total DNA and radiolabelled thymidine incorporation into DNA.

Total DNA: (West et al 1985)

This method involves the flurometric estimation of solubilised DNA. The medium was removed and the cell layer washed twice with one ml of PBS´. The DNA was solubilised by adding 1.4 mls of 10 mM EDTA adjusted to pH 12.3 with NaOh and incubation at 37°C for 20 mins. The samples were cooled on ice and the pH corrected to 7.0 by addition of 50-75 µl 1M KH_2PO_4. Immediately prior to measurement of fluorescence 1.5 ml of Hoescht 33258 solution was added to each well. After transferring to a cuvette (cat. no. 67.755 Sarstedt, Leicester, UK) fluorescence was measured using a Luminescence Spectrometer L5-3B (Perkin Elmer Ltd. Bucks. UK) with the excitation and emission wavelengths set at 350 and 450 nm respectively. Both blanks and DNA standards were run with each assay.

Radiolabelled thymidine incorporation:

Cells were incubated for 24 hours in one ml of medium containing 5 µCi [methyl-^{3}H]thymidine (5 Ci/mmol). The incubation was terminated by removing the labelled medium and washing the cell layer three times with one ml of PBS´ containing 1 mM non-radioactive thymidine. The cells were then detached by incubating for 30 mins at 37°C in one ml of trypsin/EDTA solution, supplemented with 1 mM non-radioactive thymidine. The cell suspension was transferred to 4.5 ml polypropylene tubes. The wells were then washed twice with 0.5 ml ultra-pure water containing 1 mg/ml of bovine serum albumin. Each washing was pooled with the appropriate centrifuge tube. The protein and DNA was precipitated by adding

75% (w/v) trichloroacetic acid (TCA) to a final concentration of 7.5% and the contents left overnight at 4°C. The following day the tubes were centrifuged at 1,600 x g for 20 mins in an International Electric Company Centra 7R centrifuge (Rotor 210). The supernatant was discarded and the precipitate washed with one ml of cold 7.5% TCA. Following reprecipitation for 2 hrs at 4°C the tubes were centrifuged and the supernatant was again discarded. This process was repeated one more time and the final precipitate dissolved in 0.5 ml of 0.5 M NaOH, transferred to a scintillation vial and 4.5 mls of scintillin (Picu-fluor 40) was added. The vial was then mixed and ^{3}H thymidine counted in a United Technologies Packard 2000CA liquid scintillation analyser.

Total Cell Layer Protein: (Bradford, 1976)

Total cell layer protein was assessed by colourimetric assay using Coomassie Brilliant Blue G-250. After washing twice with 1 ml of PBS the cell layer was solubilised in 0.5 ml of 0.2% (v/v) Nonident P-40 (NP-40). After scraping to ensure complete removal of the cell layer the protein solution was transferred to an Eppendorf tube and the well washed with a further 0.5 ml of NP-40. This was then pooled to the appropriate tube. The solutions were snap frozen in liquid nitrogen and stored at -70°C until required. When thawed the samples were sonicated on ice for 5 secs using the microtip of a Branson sonifer (Life Science Labs., Luton Bedfordshire, UK) at 20% of maximum output power. Protein content was then determined by adding a 50 µl aliquot to 2.5 mls of the protein assay solution. This solution contained 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (v/v)

ethanol and 8.5% (w/v) phosphoric acid. The result was determined spectrophotometrically (system 2600 Guilford Instrument Lab. Inc., Ohio, USA) at 595 nm wavelength. The values obtained were compared to a range of standards (1-25 µg) prepared from bovine serum albumin in 0.2% NP-40. The final result for one well was the mean of three replicates from that well.

Alkaline Phosphatase Activity:

Alkaline phosphatase activity was determined by measuring the release of p-nitrophenol from p-nitrophenyl phosphate as described in Sigma technical bulletin No.104 (Sigma Ltd, Dorset, UK). The samples used to determine the total cell layer protein content were also used to measure alkaline phosphatase activity. An aliquot of 100 µl was added to 900 µl of assay buffer. The assay buffer was prepared by dissolving 40 mg of p-nitrophenyl phosphate disodium in 10 mls of 221-alkaline buffer solution (2-amino-2 methyl-1 propanol buffer 1.5 mol/l pH 10.3 at 25°C) and adding 20 mls of ultra-pure water. The samples were then incubated for 30 mins at 37°C and the reaction terminated by the addition of one ml of 1 M NaOH (final concentration of 0.5 M). The amount of p-nitrophenol released was determined using a spectrophotometer (system 2600) at 410 nm wavelength. The results were compared to a standard curve which was calculated by diluting known amounts of p-nitrophenol standard solution (10 µmol/ml) with 1 ml assay buffer and 1 ml 1 M NaOH to give final concentrations of 2.5-50 nmol/ml.

The results were expressed as total alkaline phosphatase activity, alkaline phosphatase / µg protein and alkaline phosphatase / µg of DNA.

Osteocalcin:

Osteocalcin release into the medium was determined by radioimmunoassay. The cells were cultured for 24 hrs in one ml of medium which contained 2% FCS as well as the additives being tested. As the release of osteocalcin is dependent on vitamin D and its synthesis on vitamin K the media were further supplemented with 10^{-8} M $1,25(OH)_2$ Vitamin D_3 and 10^{-8} M Vitamin K. To determine the background osteocalcin present in the FCS used for each experiment one ml of medium was incubated for 24 hrs in wells without cells and harvested at the same time as those with cells. After removal the medium was frozen in liquid nitrogen and stored at -70°C until required. Osteocalcin was measured by using an osteocalcin radioimmunoassay kit (OSTK-PR, CIS Bio International, Cedex, France). The result for each well was obtained by subtracting the value obtained from the media samples incubated in the absence of cells.

Collagenase Digestion Assay: (Peterkofsky et al. 1982)

This assay was used to determine the amount of collagen and non-collagenous protein synthesised during a 24 hr labelling period. The cell layer and medium were assayed separately and the total determined by addition of these two fractions.

The cells were labelled for 24 hrs with L-[5-^{3}H] proline (23 Ci/mmol) 10 µCi/ml of medium. At the end of the incubation the medium was removed and added to 4.5 ml polypropylene tubes. The cell layer washed twice with 0.5 ml of PBS´ containing 1 mM proline and each of the washes were added to the appropriate tube. The cell layer was removed by scraping with a rubber policeman into one ml of PBS´. The cell suspension was then removed and added to separate polypropylene tubes.

The well was washed two more times with 0.5 ml of PBS which was pooled with the cell suspension from that well. Both the media and cell layer samples were frozen in liquid nitrogen and stored at -70°C until required.

After thawing the samples were sonicated on ice for 5 secs using the microtip of a Branson sonifier at 20% maximum output power. The protein was then precipitated by adding 2 mls of 20% TCA supplemented with 1 mM of proline to each tube which was then left at 4°C overnight. The precipitate was then pelleted by centrifugation at 1600 x g at 4°C for 30 mins. The supernatant was discarded and the pellet resuspended in 1 ml of 5% TCA containing 1 mM proline and left for 60 mins at 4°C. The TCA wash was repeated one more time and the pellet solubilised in 400 µl 0.2 M NaOH. The pH was partially neutralised by addition of 300 µl 0.15 M HCl and 200 µl 1M HEPES pH 7.2.

The samples were then divided in half by transfer of 450 µl to an additional tube to create two sets of tubes for each sample. The tubes were then incubated for 2 hrs at 37°C following the addition of 50 µl of collagenase-buffer solution to each tube. In one set the collagenase-buffer solution contained 2.5 Iu/tube of highly purified clostridial collagenase and the second set contained buffer alone. The buffer solutions with and without collagenase were prepared as shown in Table 1.

Table 1 Preparation of Collagenase-buffer solutions

Solutions	+ Collagenase	- Collagenase
$CaCl_2$ (25 mM)	625 µl	625 µl
N-Ethyl maleimide (62.5 mM pH 6)	1250 µl	1250 µl
Tris-HCl (0.05 M pH 7.6) + $CaCl_2$ (5 mM)	625 µl	675 µl
Collagenase solution	50 µl	-

The collagenase solution was prepared by dissolving 2,500 Iu of the highly purified collagenase (EC in one ml of Tris-HCl (0.05 M pH 7.6)) containing 5 mM $CaCl_2$ and storing in 50 µl aliquots at -70°C until required.

After two hrs incubation the tubes were placed on ice, 500 µl of cold 10% TCA containing 0.5% tannic acid added to each tube and left overnight at 4°C. The precipitate was pelleted by centrifuge at 1,600 x g for 30 mins and the supernatant transferred to 20 ml scintillation vials. The TCA wash was repeated by adding 0.5 mls 5% TCA containing 0.25% tannic acid to each pellet, mixing, and then repeating the centrifugation step. The supernatants were then pooled and 15 mls of scintillin (Pico-fluor 40) added. The pellets were dissolved by incubation with 750 µl of 1 M NaOH at 60°C for 30 mins and then transferred to a

20 ml scintillation vial and the pH neutralised with 750 µl of 1 M HCl and 15 mls of scintillin added.

After counting the collagen and non-collagenous protein synthesis was determined. The collagen component was contained within the supernatant and was calculated by subtracting the result obtained from the samples that were not treated with collagenase (i.e. background) from those that were (i.e. background + collagen). The non-collagenous protein was contained within the pellet in the collagenous treated samples.

The substrate specificity of the collagenous preparation used was assessed by determining its activity against tryptophan-labelled protein. This amino acid does not occur in collagen. Cells were incubated for 24 hrs in one ml of medium containing 250 µmol of Na ascorbate and 10 µCi of L-[G-^{3}H]tryptophan (7.2 Ci/mmol). At the end of this period the cell layer and medium were processed as described for L-[5-^{3}H] proline. After subtracting the background from the collagenase treated samples for six replicates, 0.4 (± 0.02)% of the tryptophan was released from the cell layer and 0.1 (± 0.01)% from the medium confirming that the preparation is largely free of non-specific proteolytic activity.

RESULTS

Primary Culture:

Proliferation

The cells in ASC 2P supplemented standard medium reached confluence sooner and there was a greater than 3 fold increase in cell number when the cells were passaged.

Explant Histology

The light microscopic examination of explants

cultured in standard medium showed few cells and only the occasional cell staining positive for alkaline phosphatase. These cells appeared to be located in a thin rim around the margins of the explant. There was a sparse matrix and the stromal architecture was not preserved. Explants cultured in ASC 2P supplemented medium contained more cells many of which were alkaline phosphatase positive. There was extensive matrix formation and the tissue architecture appeared to be well preserved

Crossover Study:

Cell proliferation

Both the DNA and thymidine incorporation studies demonstrated an increased proliferative capacity of cells cultured in ASC 2P in primary culture. Proliferation was decreased when ASC 2P was withdrawn but it still remained higher than in cells never exposed to ASC 2P. When comparing SS cells with AA from primary through to the end of the secondary culture there is a ten fold increase in cell number (see Figs. 2 and 3).

Alkaline Phosphatase Activity

Alkaline phosphatase activity is associated with mineralisation and appears to be largely confined to bone cells that are actively synthesising matrix. It is therefore a useful marker of differentiation.

The cells cultured in ASC 2P in primary culture had reduced alkaline phosphatase activity when corrected for protein or DNA. The alkaline phosphatase activity was increased when ASC 2P was withdrawn in secondary culture (see Figs. 5a, b, and c).

Osteocalcin Release

Osteocalcin is one of the non-collagenous proteins of bone and is probably the only one that is unique to calcified tissue.

In groups not treated with $1,25(OH)_2VitD_3$ and vitamin K there was a small base line level of osteocalcin which varied between 1-3 ng/ml. Following correction for either total protein or DNA these levels were constant between each of the groups. After 24 hrs incubation with D and K the total osteocalcin released was greatest in the AA treated group. This difference with the other culture conditions was also maintained when corrected for total protein and DNA. The SA treated group was also significantly increased when corrected for DNA but not for protein. The AS treated group was also elevated compared to SS except when corrected for protein (Figs. 6a, b and c).

Collagen synthesis and Non-collagenous protein synthesis

The extracellular matrix of bone constitutes 92-95% of the total volume of bone. It consists of both organic (22% by weight) and inorganic (69%) components. The organic matrix is largely collagen (90%) and the remainder is the heterogeneous group of non-collagenous proteins.

a) Collagen Synthesis

In the absence of ASC 2P in secondary culture there is very little collagen formed in both groups. Total collagen synthesis is highest in the AA treated group and after correcting for DNA there is a 50% increase in the AA compared to SA treated groups. The difference between AA and AS groups is greatest in the cell layer (see Figs. 7a, b and c).

b) Non-collagenous Protein Synthesis

The changes in non-collagenous protein synthesis have a similar pattern to that seen in the collagen synthesis results. However ASC 2P in secondary culture does not make as great a difference to non-collagenous protein synthesis as it does to collagen synthesis (see Figs. 8a, b and c).

Effects of Vitamins D & K and dexamethasone

Vitamin D is essential for normal mineralisation. Vitamin K is necessary in the formation of non-collagenous proteins of bone matrix.

On Total Alkaline Phos. Activity

If ASC was present then total alkaline phosphatase was increased by the addition of Dexamethasone and D+K either alone or in combination. The percentage increase was greatest in the AA group particularly the increase due to D+K. In the absence of ascorbate the cells have a reduced response to both D+K and dexamethasone (see Figs. 9a, b and c).

CONCLUSION

The presence of ASC 2P in primary culture is necessary for the maintenance of stromal integrity within the explants. In addition it alters the cell population obtained by outgrowth from the explants. Cells which have been in the continual presence of ASC 2P proliferate faster and synthesise more collagen and non-collagenous proteins. They have a reduced amount of alkaline phosphatase but this can be readily induced with either $1,25(OH)_2VitD_3$ or dexamethasone. Osteocalcin release following stimulation with $1,25(OH)_2VitD_3$ is high.

In further studies (results not shown)

mineralisation and adipocytic differentiation were observed using light and electron microscopy. Mineralisation was seen only in AS and AA groups (also containing dexamethasone, vitamins D and K and β-glycerophosphate). Adipocytic differentiation occurred in all cultures containing dexamethasone that had been treated with ASC 2P in primary culture.

These findings are consistent with the conclusion that the presence of long-acting ascorbate in primary culture results in increased numbers of cells which are less differentiated but are capable of differentiation to functional osteoblasts and adipocytes.

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LEGENDS TO THE DRAWINGS

Figure 2

Effects of ASC 2P in primary culture on total DNA.

Results expressed as the mean, n = 3, error bars = SEM; ** = $p < 0.01$ and *** = $p < 0.001$ compared to control containing vehicle only.

= $p < 0.001$ compared to AA treated culture

SS = standard medium in both primary and secondary culture.

SA = standard medium in primary but 100 µM ASC 2P in secondary.

AS = ASC 2P in primary culture and standard medium in secondary.

AA = ASC 2P in both primary and secondary culture.

Figure 3

Effects of ASC 2P in primary culture on total thymidine incorporation.

Results expressed as the mean, n = 3, error bars = SEM; ** = $p < 0.01$ and *** = $p < 0.001$ compared to control (SS) containing vehicle only.

= $p < 0.001$ compared to AA treated culture

SS = standard medium in both primary and secondary culture.

SA = standard medium in primary but 100 µM ASC 2P in secondary.

AS = ASC 2P in primary culture and standard medium in secondary.

AA = ASC 2P in both primary and secondary culture.

Figure 4

Effects of ASC 2P in primary culture on total protein content.

Results expressed as the mean, n = 3, error bars = SEM; ** = $p < 0.01$ and *** = $p < 0.001$ compared to control (SS) containing vehicle only.
= $p < 0.001$ compared to AA treated culture
SS = standard medium in both primary and secondary culture.
SA = standard medium in primary but 100 µM ASC 2P in secondary.
AS = ASC 2P in primary culture and standard medium in secondary.
AA = ASC 2P in both primary and secondary culture.

Figure 5a

Effects of ASC 2P in primary culture on total alkaline phosphatase activity.
1 unit = 1 nmol of p-nitrophenol released / 30 mins / ml.
Results expressed as the mean, n = 3, error bars = SEM; ** = $p < 0.01$ and *** = $p < 0.001$ compared to control (SS) containing vehicle only.
= $p < 0.001$ compared to AA treated culture
SS = standard medium in both primary and secondary culture.
SA = standard medium in primary but 100 µM ASC 2P in secondary.
AS = ASC 2P in primary culture and standard medium in secondary.
AA = ASC 2P in both primary and secondary culture.

Figure 5b

Effects of ASC 2P in primary culture on total alkaline phosphatase activity /total protein content.

Figure 5c)

Effects of ASC 2P in primary culture on total alkaline phosphatase / DNA.
1 unit = 1 nmol of p-nitrophenol released / 30 mins / ml.
Results expressed as the mean, n = 3, error

bars = SEM; ** = p < 0.01 and *** = p < 0.001, ### = p < 0.001 .

Figure 6a

Effects of ASC 2P in primary culture on osteocalcin production.

Results expressed as the mean, n = 3, error bars = SEM; *** = p < 0.001 compared to control (SS) ### = p < 0.001 compared to AA treated culture
SS = standard medium in both primary and secondary culture.
SA = standard medium in primary but 100 µM ASC 2P in secondary.
AS = ASC 2P in primary culture and standard medium in secondary.
AA = ASC 2P in both primary and secondary culture.

Figure 6b

Effects of ASC 2P in primary culture on osteocalcin release / total protein content.

Figure 6c)

Effects of ASC 2P in primary culture on osteocalcin released / total DNA.
SS + standard medium alone, SA standard medium in primary and ASC 2P in secondary, AS = ASC 2P in primary but standard medium in secondary, AA = ASC 2P in both primary and secondary.

Results expressed as the mean, n = 3, error bars = SEM; * = p < 0.05, ** = p < 0.01 and *** = p < 0.001 compared to SS. # = p < 0.05, ## = p < 0.01, ### = p < 0.001 Compared to AA.

Figure 7a

Effects of ASC 2P in primary culture on total collagen synthesis. Results expressed as the mean,

n = 3, error bars = SEM; *** = p < 0.001 compared to control containing vehicle only, ### = p < 0.001 compared to AA.

Figure 7b

Effects of ASC 2P in primary culture on collagen synthesis in the media layer and

Figure 7c

Effects of ASC 2P in primary culture on collagen synthesis in the cell layer.

Results expressed as the mean, n = 3, error bars = SEM; *** = p < 0.001 compared to control containing vehicle only, ### = p < 0.001 compared to ASC 2P.

Figure 8a

Effects of ASC 2P in primary culture on total non-collagen protein synthesis.

Results expressed as the mean, n = 3, error bars = SEM; *** = p < 0.001 compared to control containing vehicle only, ### = p < 0.001 compared to AA

Figure 8b

Effects of ASC 2P in primary culture on non-collagenous protein synthesis in the media layer and

Figure 8c

Effects of ASC 2P in primary culture on non-collagenous protein synthesis in the cell later.

Results expressed as the mean, n = 3, error bars = SEM; * = p < 0.05, ** = p < 0.01, *** = p < 0.001 compared to SS. # = p < 0.05, ## = p < 0.01, ### = p < 0.001 Compared to AA.

Figure 9a

Effects of ASC 2P in primary culture on total alkaline phosphatase activity in combination with DEX,

VIT D or both.

Figure 9b

Effects of ASC 2P in primary culture on alkaline phosphatase / total protein in combination with Dex, Vit D, or both.

Figure 9c

Effects of ASC 2P in primary culture on Alkaline phosphatase / DNA in combination with Dex, Vit D, or both.

CLAIMS

1. A method of maintaining bone and bone-forming cells in culture wherein the cells are provided with a long-acting source of ascorbate from the primary culture stage.

2. A method as claimed in claim 1 where the long-acting form of ascorbate is L-ascorbic acid 2-phosphate.

3. A method as claimed in claim 1 or claim 2 wherein the bone and bone-forming cells are human.

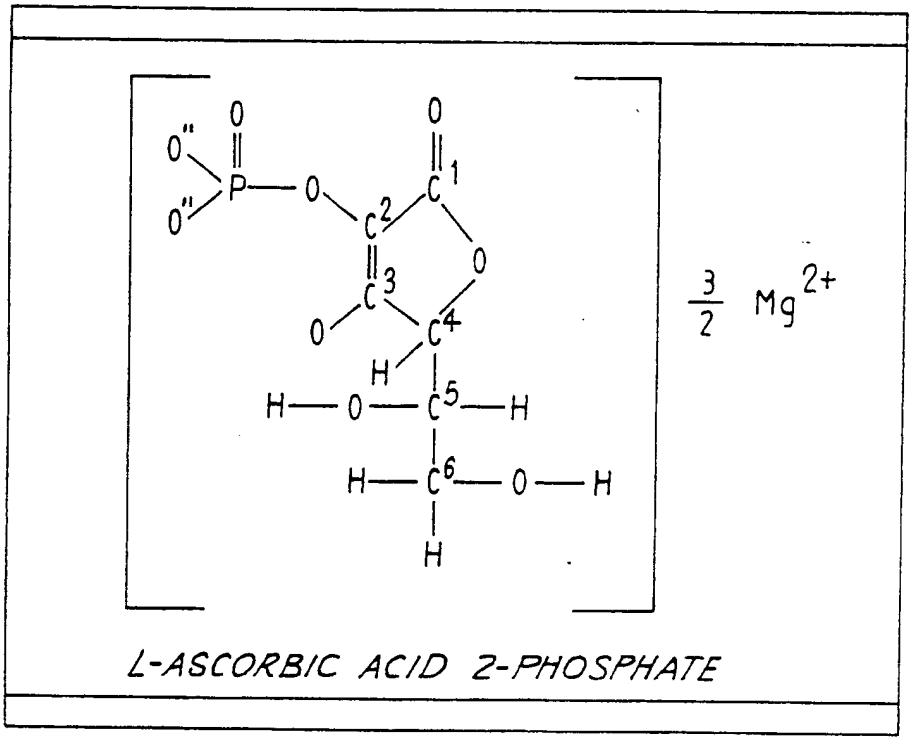
4. A method as claimed in any one of claims 1 to 3 wherein the cultured cells are implanted as an allograft or an autograft or a xenograft.

5. A method as claimed in claim 4 wherein the cultured cells are implanted with the support of a biocompatible matrix.

6. A method as claimed in claim 4, wherein the graft is in the form of a suspension of cells introduced into the body.

7. A method as claimed in any one of claims 1 to 6, wherein one or more artificial materials are added to the cells and the effect of the material or materials on the growth and condition of the cells is assessed.

FIG. 1



L-ASCORBIC ACID 2-PHOSPHATE

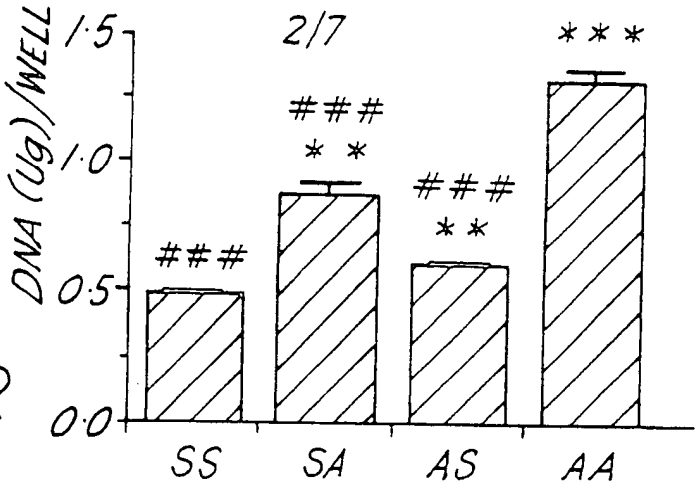


FIG. 2

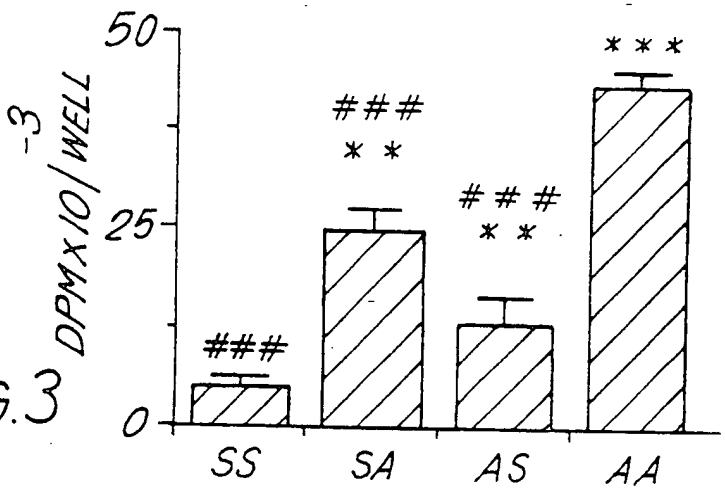


FIG. 3

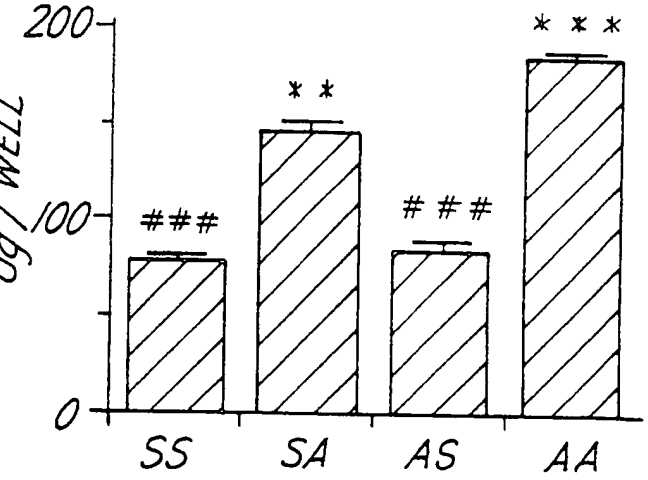


FIG. 4

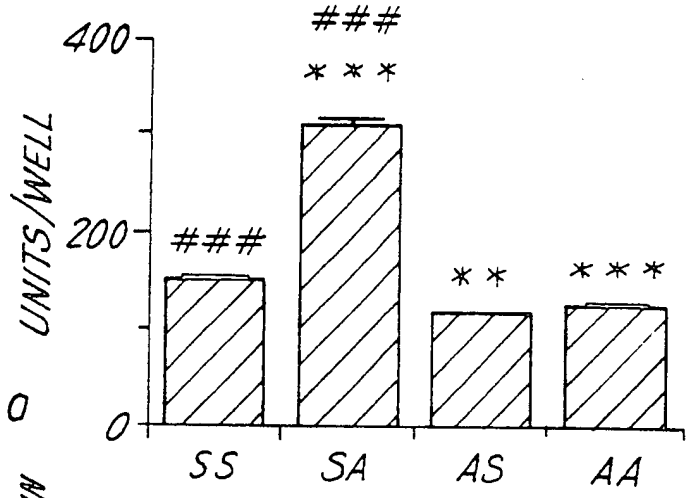


FIG.5a

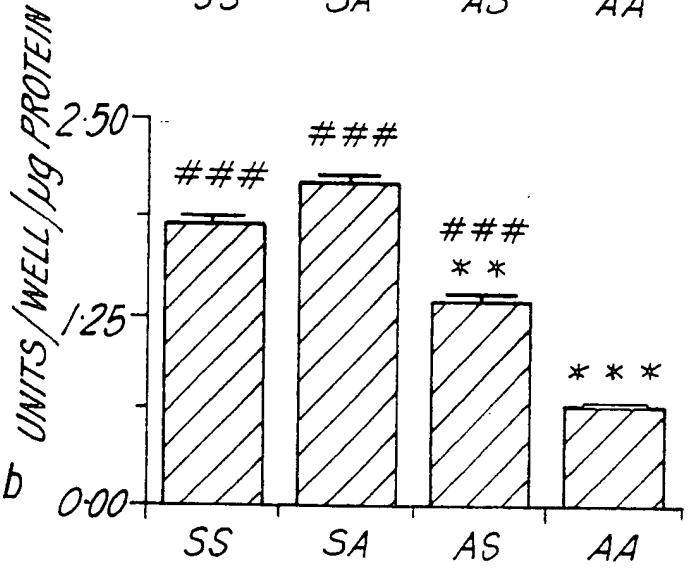


FIG.5b

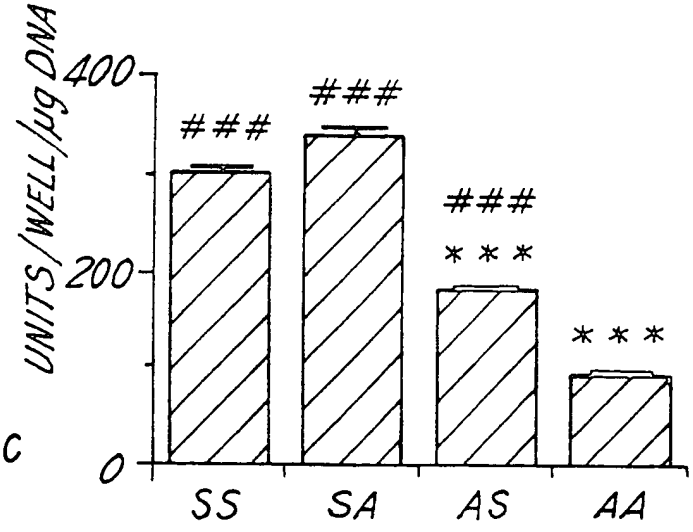


FIG.5c

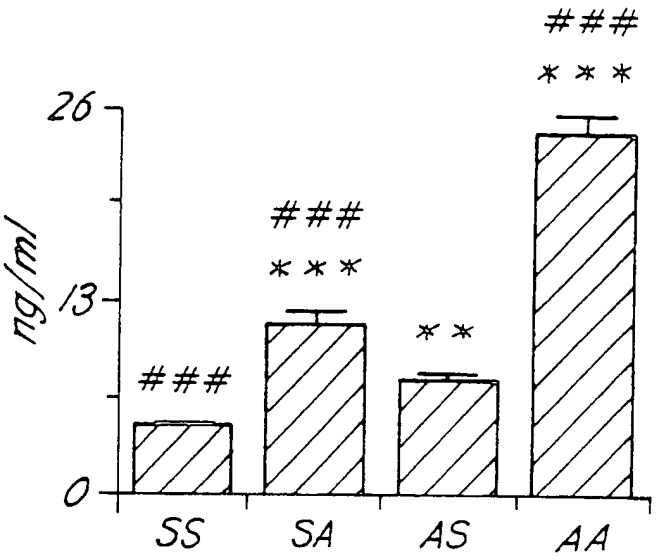


FIG.6a

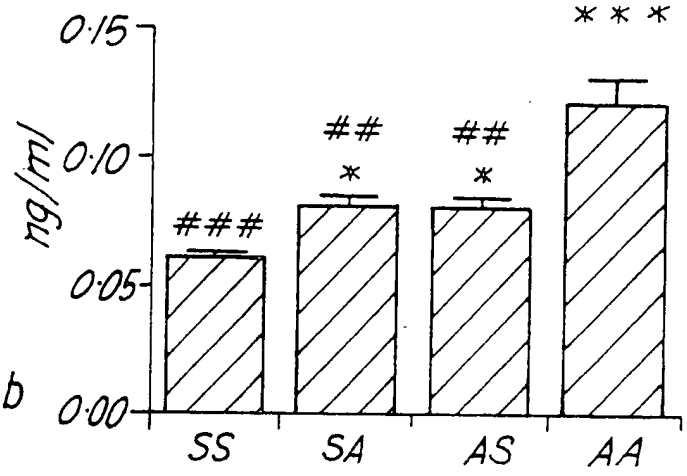


FIG.6b

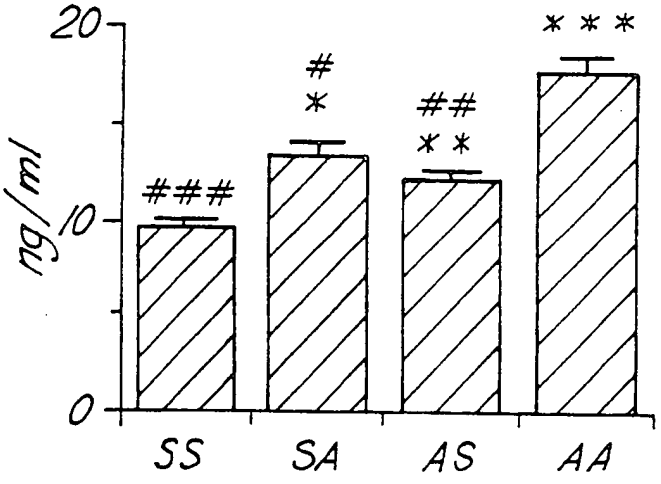


FIG.6c

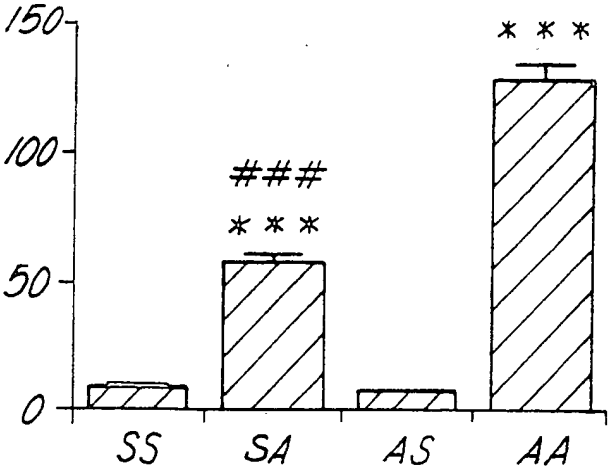


FIG. 7a

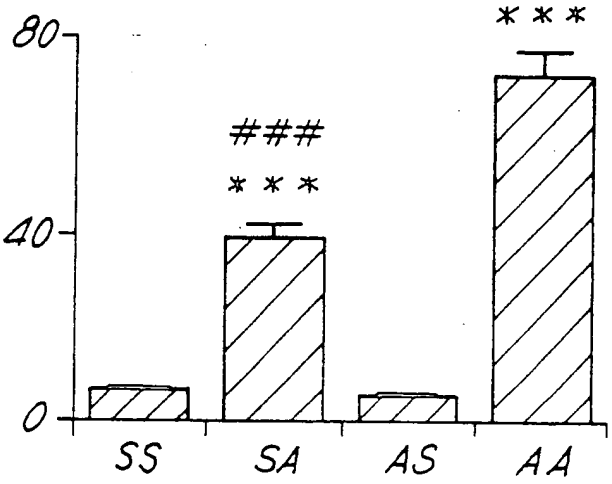


FIG. 7b

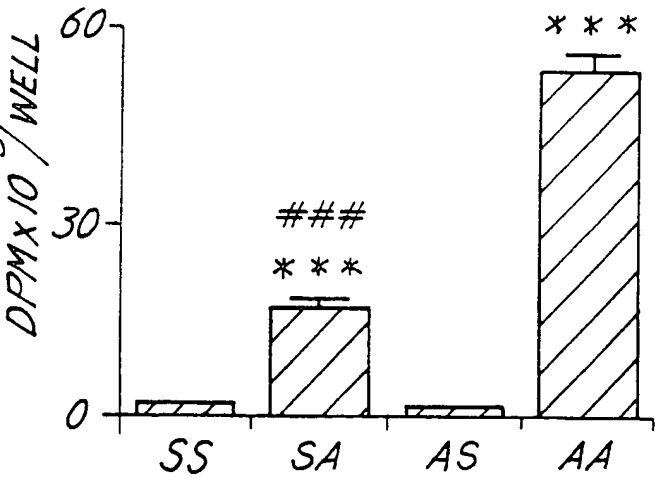


FIG. 7c

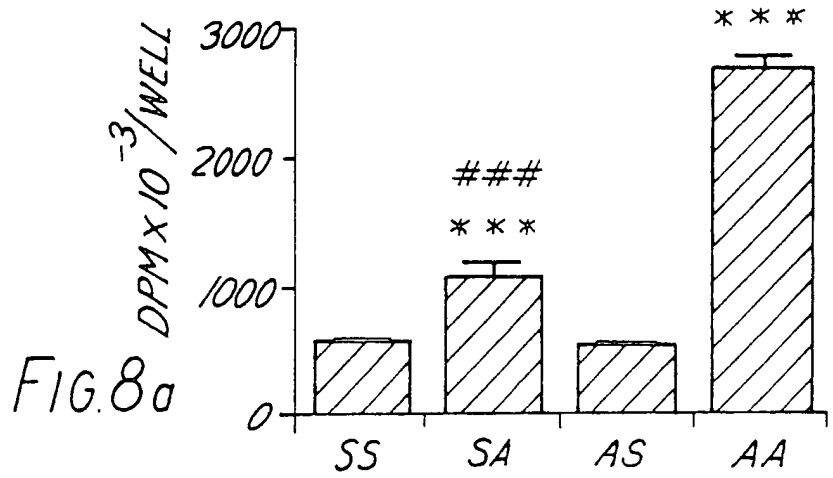


FIG. 8a

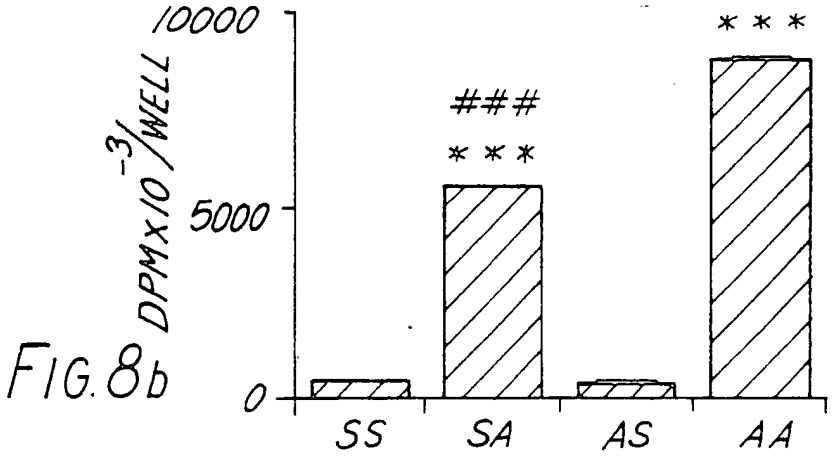


FIG. 8b

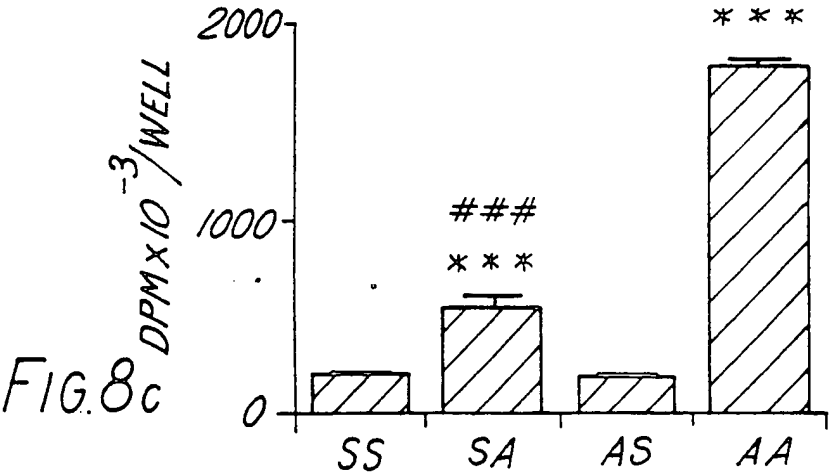


FIG. 8c

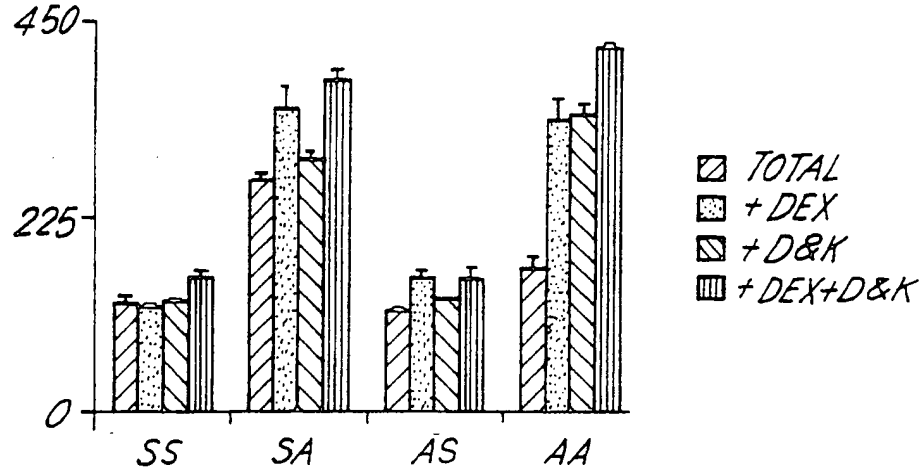


FIG. 9a

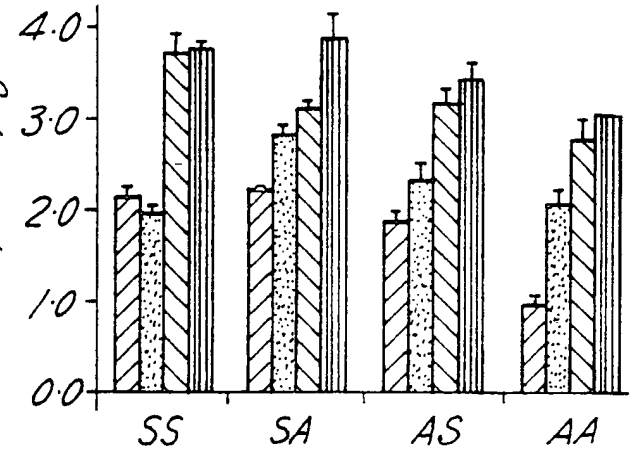


FIG. 9b

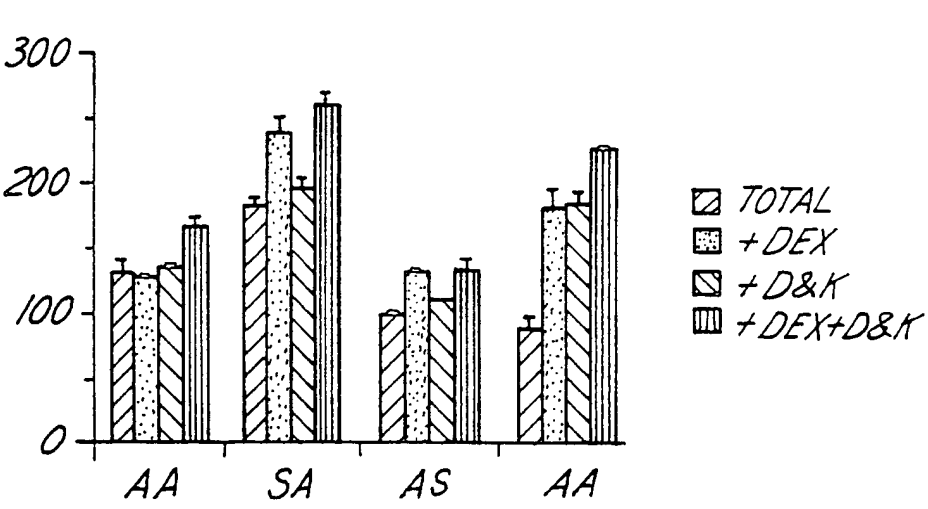


FIG. 9c

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/02185

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N5/08; A61L27/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A61K ; A61L

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	EP,A,0 282 746 (TAKEDA CHEMICAL INDUSTRIES, LTD.) 21 September 1988 see page 3, column 3, line 38 - line 58; claims see page 4, column 5, line 4 - line 11 see page 5, column 7, line 25 - line 32 see example 5 ---	1-7
X	BONE vol. 11, no. 5, 1990, NEW YORK, N.Y., US page 378 S.E. GRAVES ET AL. 'EFFECTS OF L-ASCORBIC ACID 2-PHOSPHATE ON HUMAN BONE DERIVED CELLS.' cited in the application see the whole document --- -/--	1-7

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17 FEBRUARY 1993	09. 03. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	RYCKEBOSCH A.O.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	EP,A,0 339 607 (S. ITAY) 2 November 1989 see page 3, column 4, line 2 - line 14; claim 1; example 1	1-7

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

GB 9202185
SA 66895

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0282746	21-09-88	JP-A-	1010983	13-01-89
EP-A-0339607	02-11-89	US-A-	5053050	01-10-91
		US-A-	4904259	27-02-90
		AU-B-	625913	16-07-92
		AU-A-	3380889	02-11-89
		JP-A-	2209811	21-08-90